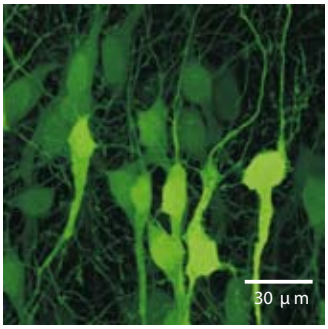
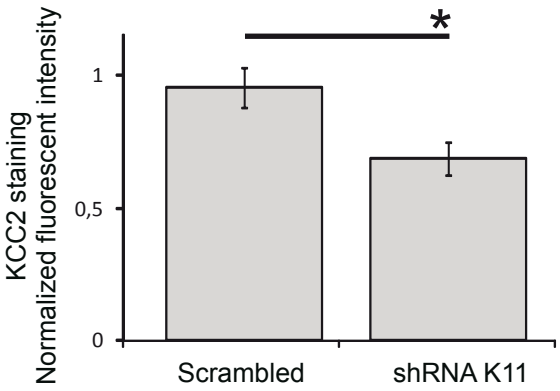
A

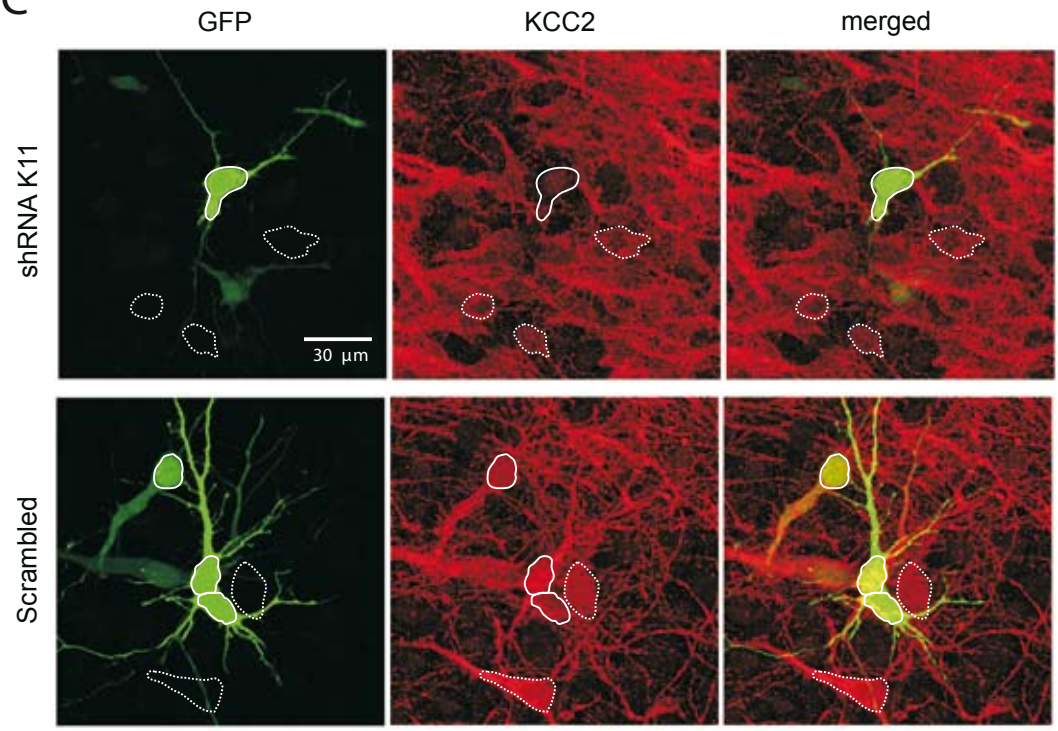


GFP electroporated cells

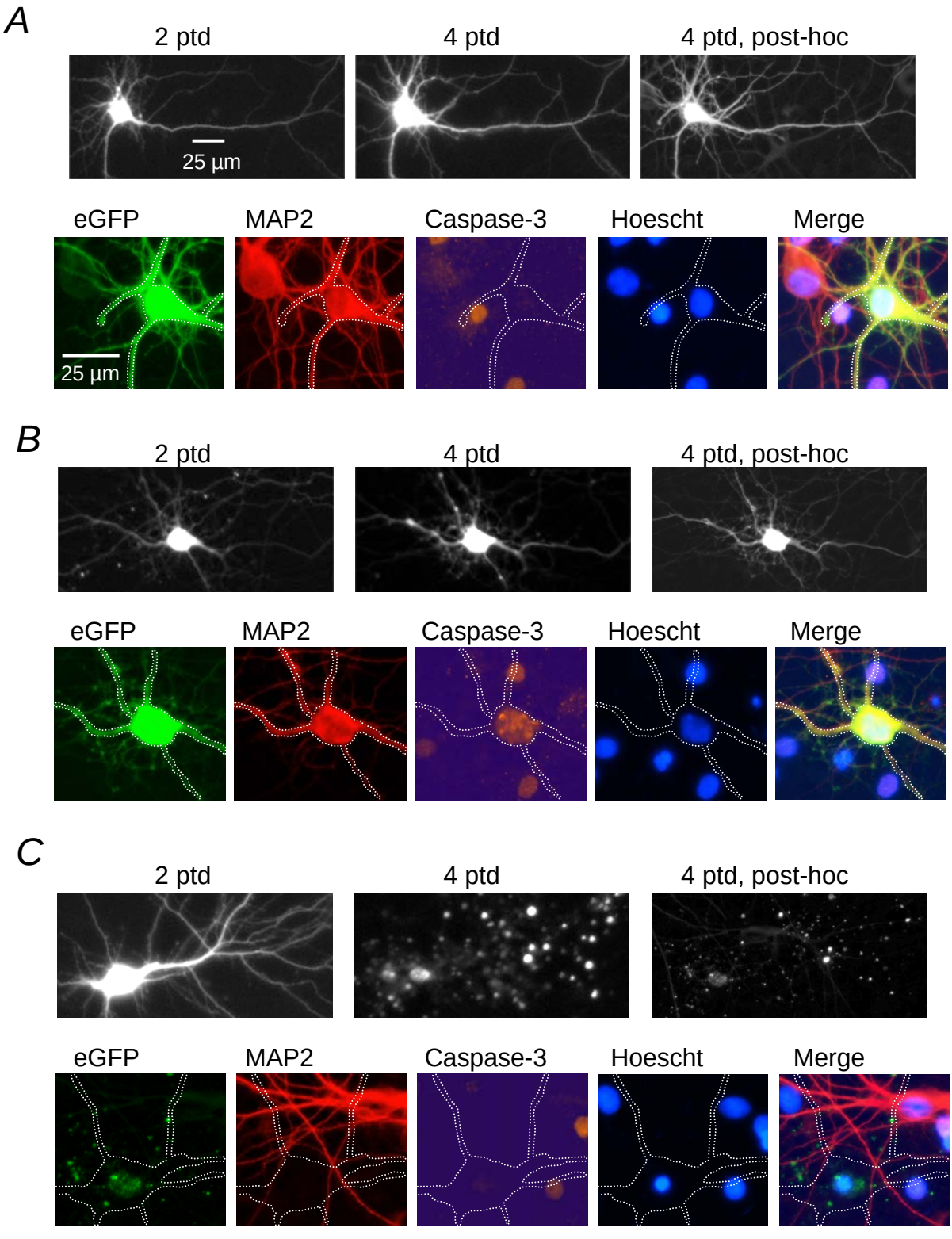
B



C



Supplementary Fig. 1, Pellegrino



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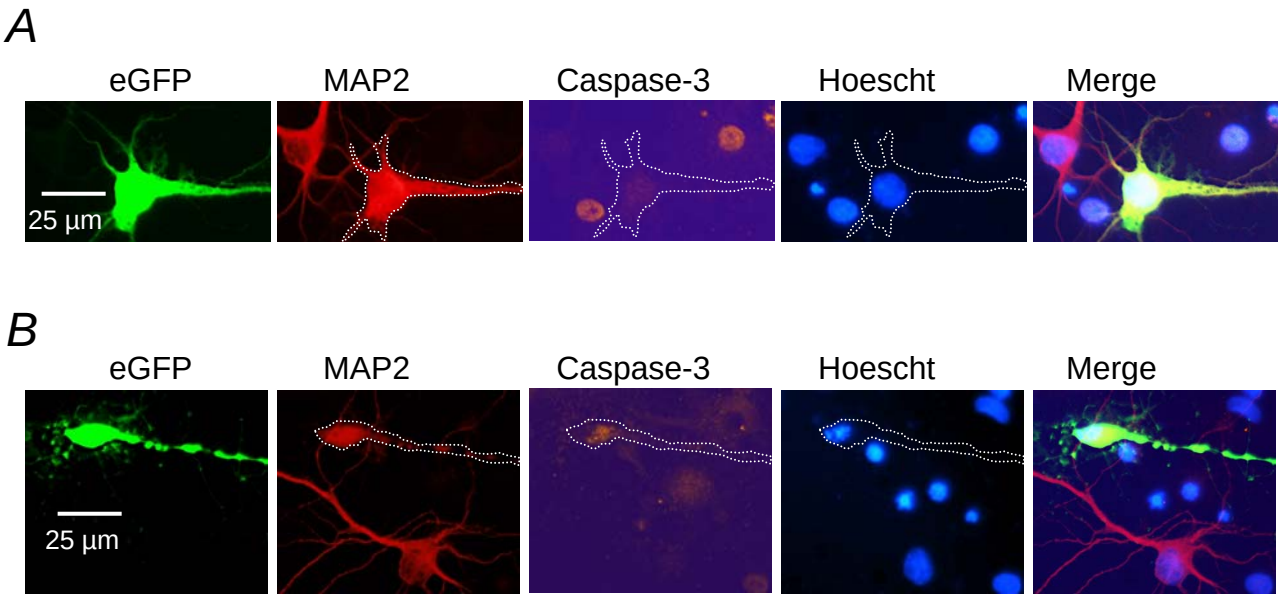

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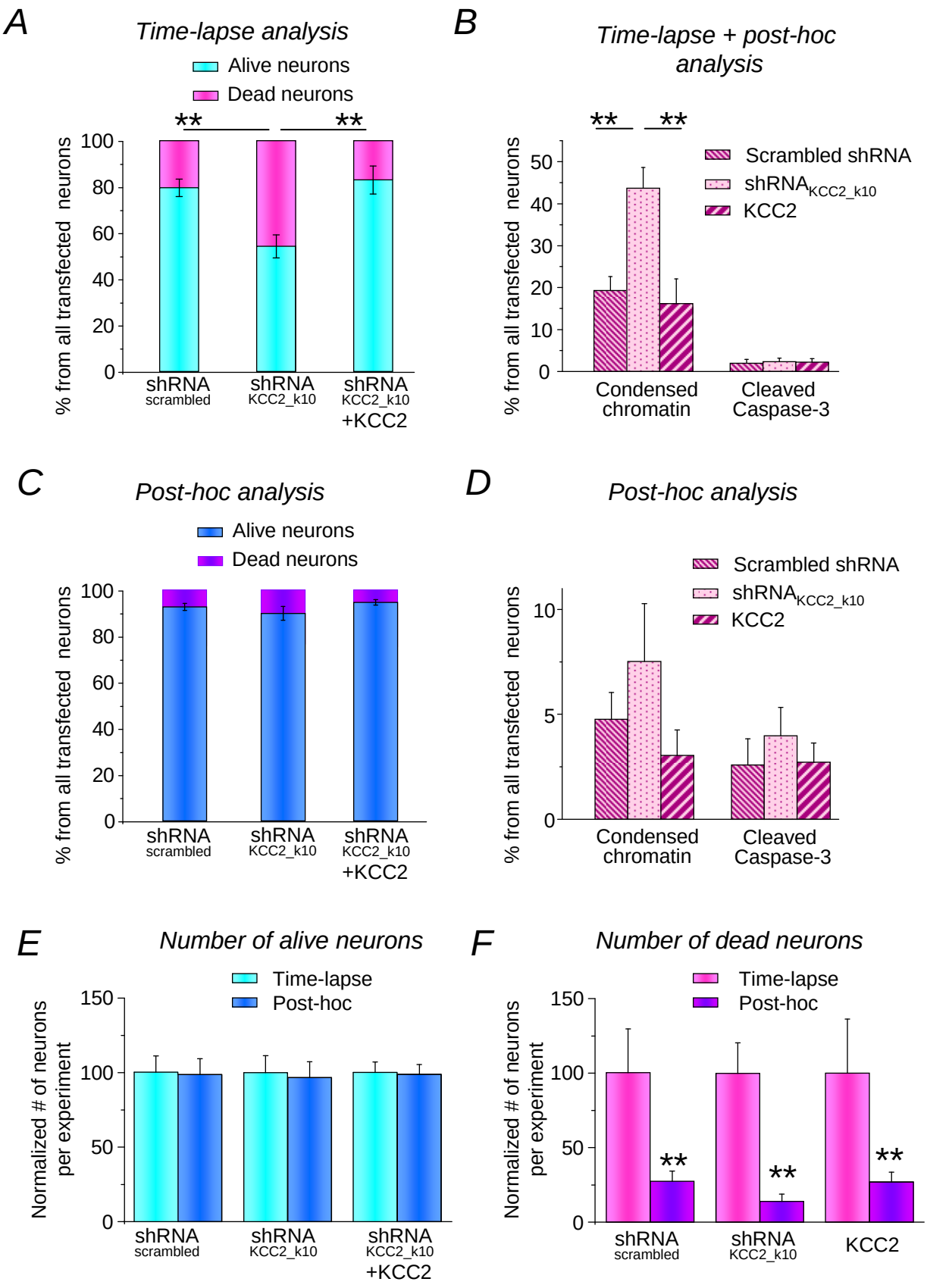

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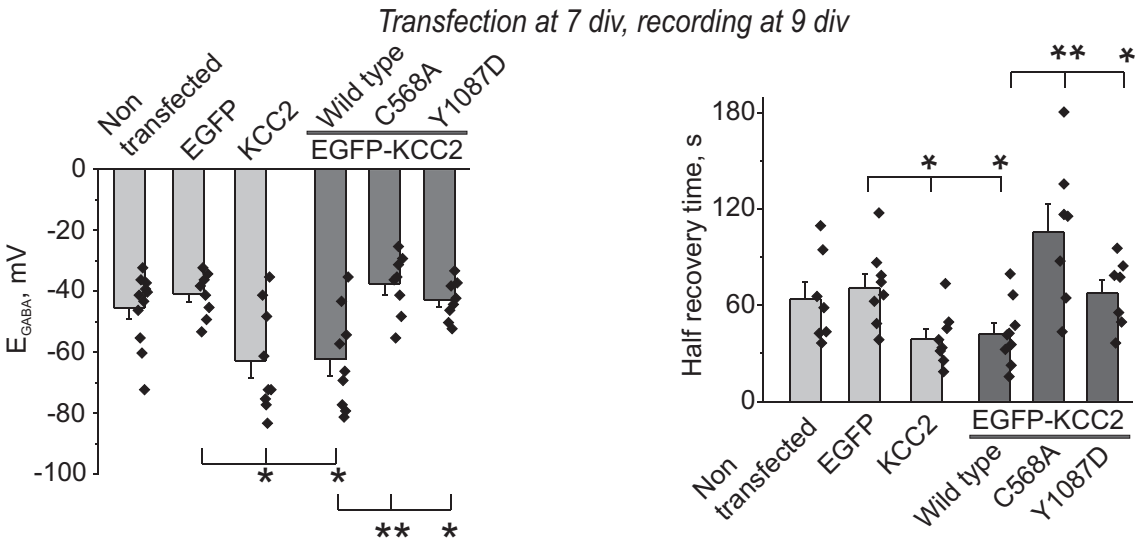
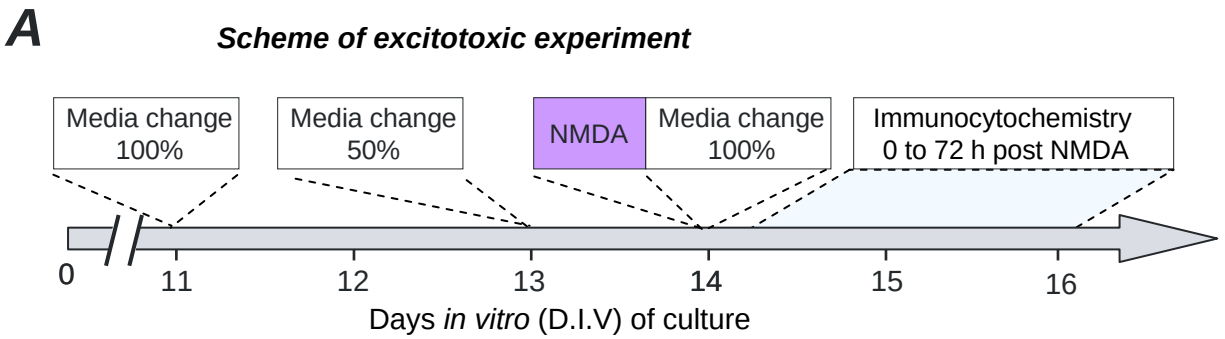
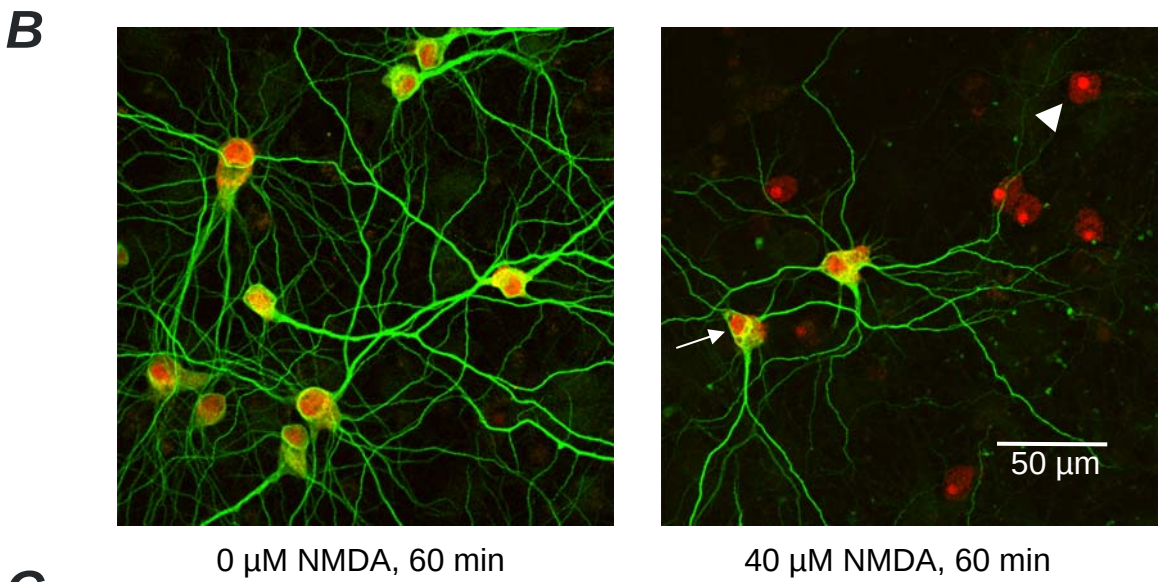
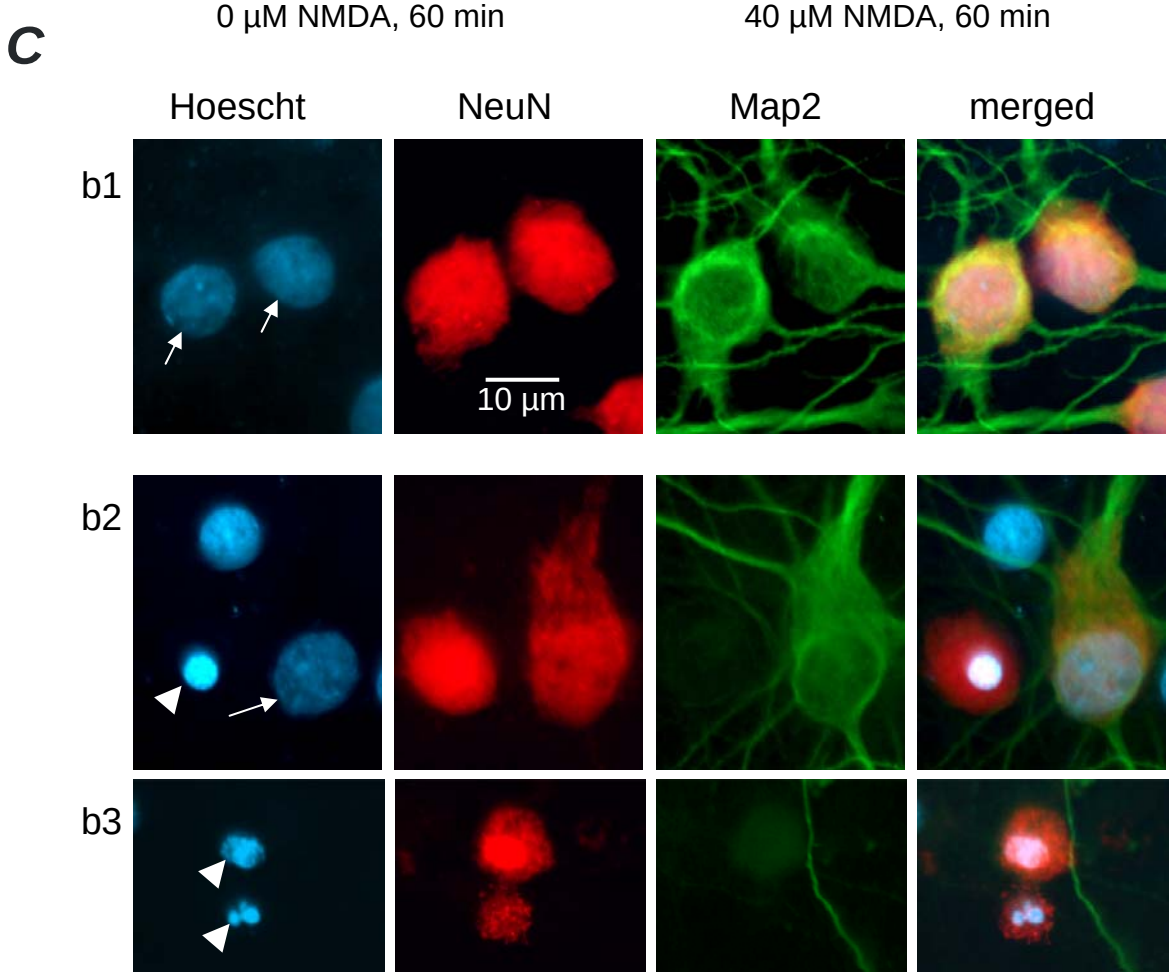


Supplementary Fig. 5, Pellegrino

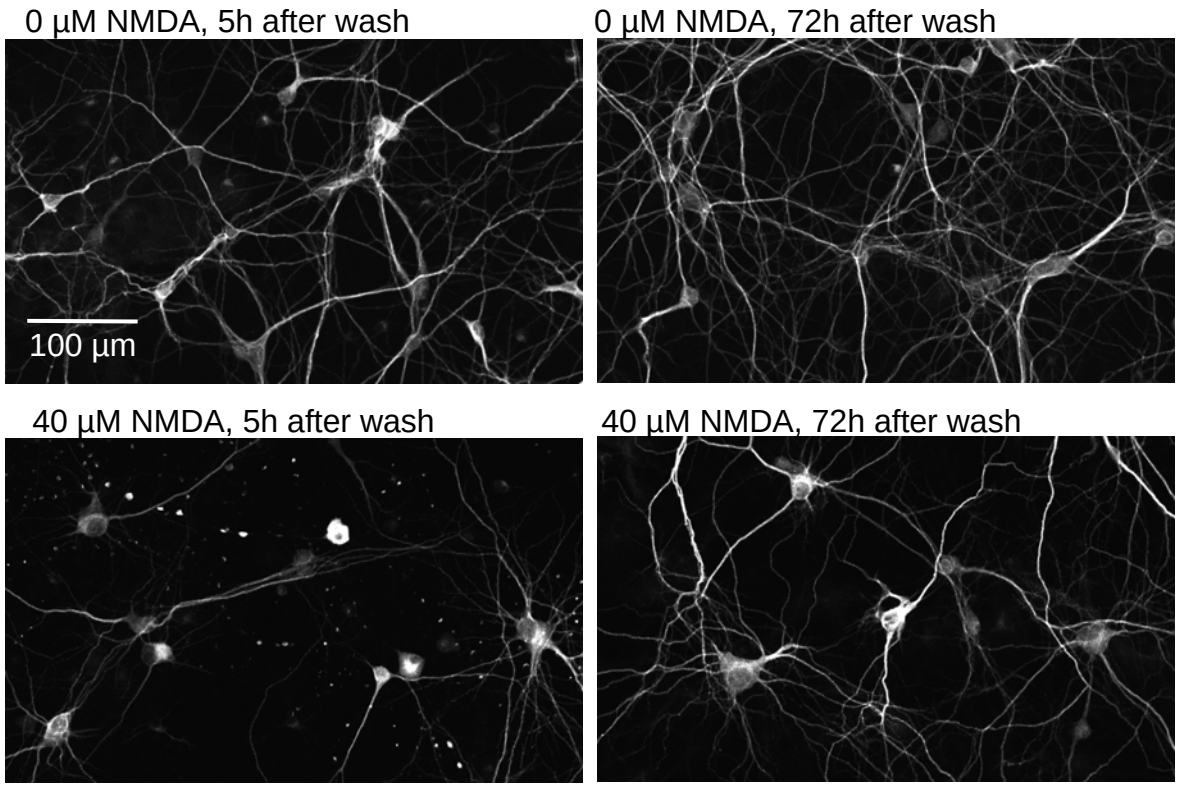




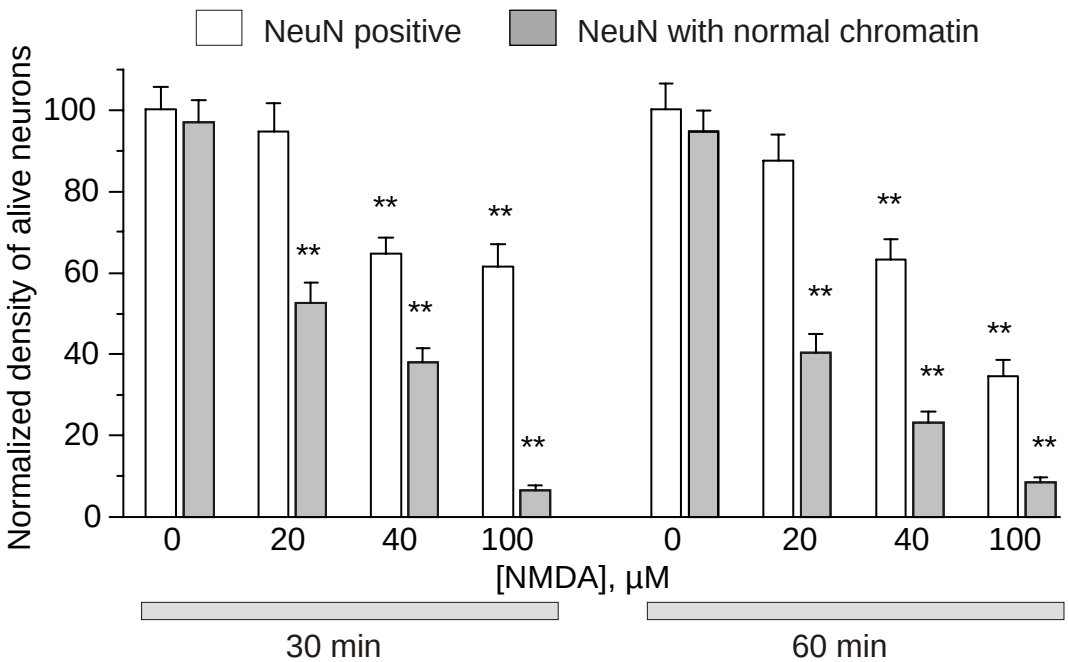


Supplementary Fig. 6. Pellegrino

A



B



Supplementary Fig. 7, Pellegrino

Supplementary material

Analysis of neuronal survival.

Supplementary Fig. 2 provides comparison of two approaches for quantification of dying and alive transfected neurons. Neuronal cultures were transfected with scrambled shRNA, $shRNA_{KCC2\_K10}$ and $shRNA_{KCC2\_K10}$ + mouse KCC2 at 9 div as described in "Methods". During 4 days after transfection the fate of transfected neurons was followed using time-lapse approach, then, the cultures were fixed and analyzed "post-hoc" using immunostaining for MAP2, a specific neuronal marker, and Cleaved Caspase-3 as well as nuclei staining with Hoechst.

"Time-lapse approach". The percentage of alive or dead transfected neurons was determined using analysis of their morphology in living cultures. As alive we considered neurons with homogeneously distributed fluorescent marker (eGFP) in dendrites and soma as well as neurons that included few puncta in dendrites, but still had clearly visible dendritic structure **(Fig. 6*A*** and **Supplementary Fig. 2*A*)**. When the same neurons were visualized after post-hoc staining, using their time-lapse coordinates, we saw that 98% of these neurons had homogeneously distributed chromatin and MAP2 staining and were Cleaved Caspase-3 negative. Only small populations of neurons classified as "alive" (1.7 to 2.5 %) were revealed with Cleaved Caspase-3 antibody, or had slightly condensed chromatin (See **Supplementary Fig. 2*B*** for example of rare Cleaved Caspase-3 positive neurons). As dead we considered two types of neurons: neurons, that disappeared from the optical field or neurons having fluorescent marker only in form of condense puncta without signs of homogeneous fluorescence distribution **(Fig. 6*A*** and **Supplementary Fig. 2C)**. Among dead neurons, which were still present at the moment of fixation, only 6 cells (out of 219 neurons denominated as dead) showed immunochytochemistry staining characteristic for alive cells. Thus, the criteria used for analysis of life images of transfected neurons provided reliable approach to determine neuronal viability.

"Post-hoc approach". The percentage of alive or dead transfected neurons in fixed cultures. For analysis were taken all transfected cells showing at least some traces of staining with MAP2. Such cells represented 99% of all transfected cells in studied samples of cultures (596 out of 604). As alive we considered neurons showing homogeneous distribution of the MAP2 staining in dendrites, homogeneous distribution of the chromatin, revealed by Hoechst staining and absence of staining with Cleaved Caspase-3 antibody. A typical example of such a neuron is shown in **Supplementary Fig. 3*A***. As dead, we considered neurons with

condensed chromatin into nuclei or presence of positive staining with Cleaved Caspase-3 antibody. A typical example of such neuron is shown in **Supplementary Fig. 3*B***.

Time-lapse approach revealed significant ($P<0.01$) decrease in the % of alive neurons and increase in the % of dead neurons expressing $shRNA_{KCC2\_K10}$ as compared to neurons expressing scrambled shRNA or $shRNA_{KCC2\_K10}$ + mouse KCC2 (**Supplementary Fig. 4*A, B)***. By contrast, traditional post-hoc analysis of the same cultures that were used for time-lapse studies did not reveal any significant change in neuronal survival/death induced by $shRNA_{KCC2\_K10}$ (**Supplementary Fig. 4*C* and *D)***.

Which difference between time-lapse and post-hoc approaches causes such a strong discrepancy in the data? Analysis showed that the number of alive neurons per experiment quantified in living cultures (time-lapse approach) and in the same cultures fixed 3 hours after life imaging (post-hoc approach) was almost identical (**Supplementary Fig. 4*E)***. By contrast, the numbers of dead neurons revealed using time-lapse and post-hoc approaches were significantly different in every experimental case (**Supplementary Fig. 4*F)***. The explication of this discrepancy is that time-lapse takes into account dead cells which are not detectable using post-hoc analysis. Example of such a neuron is given in **Supplementary Fig. 2*C.***

A transient transfection of primary neuronal cultures with exogenous cDNAs became a powerful tool of analysis of the functional role of proteins. Our results illustrate an importance of appropriate choice of method of analysis when studying small populations of transfected neurons. Time-lapse approach is one of ways allowing characterizing the fate of every genetically modified neuron, easy determining of the time point of highest effectiveness of the molecule of interest and avoiding possible artifacts related to the variability in rates of cultures transfection with different constructs.

Legends to supplementary figures.

Supplementary Figure 1. shRNAs silencing of KCC2 in rat organotypic slices.

A, an example image of GFP-electroporated neurons in cultured rat organotypic slice, maximal z-projection of 140 optical sections, 0.13μm each.

B, quantification of KCC2 immunoreactivity in shRNA transfected neurons. The value of KCC2 immunostaining intensity in neighboring non-transfected neurons is set to 1. (*, $p<0.05$, n=5-6 neurons, 2-4 slices).

C, images of transfected neurons in organotypic slices electroporated with $shRNA_{KCC2\_K11}$ + eGFP (upper row) or scrambled shRNA + eGFP (bottom row); z-projection of 30 optical sections, 0.13 μm each. Neuronal cell body is outlined. Note the low KCC2 immunoreactivity in $shRNA_{KCC2\_K11}$ transfected neurons as compared to neighboring non-transfected neurons and neurons transfected with scrambled shRNA.

Supplementary Figure 2. Post-hoc analysis of the properties of neurons determined as "alive" (*A, B*) or "dead" (*C*) using time-lapse imaging. All neurons are from the sample of culture transfected with $shRNA_{KCC2\_K10}$. Upper rows in each panel show two live images of neurons taken 2 and 4 days after transfection (ptd, post-transfection days), and image of the same neurons taken after fixation and immunocytochemical reactions. Bottom rows illustrate the soma region of neurons shown in upper row (4 days post transfection, post-hoc treatment), but revealed using MAP2 antibody, Cleaved Caspase-3 antibody or Hoechst as indicated. The white dotted lines outline contours of transfected neurons.

A, a typical alive neuron. This neuron was assigned as alive based on its morphology at 4 ptd. This neuron was MAP2 positive, Cleaved Caspase-3 negative and had homogeneously distributed chromatin in the nucleus.

B, an example of rare Cleaved Caspase-3 positive neuron. This neuron was assigned as alive based on its morphology at 4 ptd. Notice normal distribution of MAP2 immunoreactivity and homogeneous chromatin into the nuclei. Such Cleaved Caspase-3 positive neurons constituted less than 5% of all transfected neurons.

C, a typical example of dead neuron. This neuron was assigned as dead based on degradation of its morphology at 4 ptd. The traces of this neuron were localized using coordinates recorded during time-lapse imaging. It was impossible to detect the remaining of this cell by direct screening of fixed culture. Notice the presence of the condensed chromatin localized among the traces of eGFP and absence of MAP2 immunoreactivity.

Supplementary Figure 3. Examples of different types of transfected neurons used for post-hoc analysis of neuronal viability. All neurons are from the sample of cultures transfected with $shRNA_{KCC2\_K10}$ and identified by the green fluorescence of eGFP. The white dotted lines outline contours of transfected neurons.

A, a typical alive neuron revealed by combination of antibodies. The green fluorescence of eGFP is homogeneously distributed in the cell, the MAP2 staining present no visible signs of fluorescence puncta in the dendrites, the Cleaved Caspase-3 staining is also negative in this cell and finally the chromatin is not condensed as shown by the Hoechst staining in blue.

B, a typical "dead" neuron. First of all, the MAP2 staining is not homogenous, then the cleaved caspase-3 staining is positive within this cell and finally the chromatin, revealed by the Hoechst staining, is condensed.

Supplementary Figure 4. Results of the quantification of alive and dead neurons from same cultures using time-lapse and post-hoc approaches. Cultures were transfected with scrambled shRNA, $shRNA_{KCC2\_K10}$ or $shRNA_{KCC2\_K10}$ + mouse KCC2 at 9 div. Live images of all transfected neurons (24 to 67 per well) were taken on 2^{nd} and 4^{th} days after transfection. Thereafter neurons were fixed and immunostained with anti-MAP2, anti-cleaved caspase-3 antibodies and Hoechst. Mean ± SEM, 6 experiments. Asterisks indicate significant differences (*, $P<0.05$; **, $P<0.01$)

A, proportions of alive and dead neurons obtained after time-lapse analysis of the cultures. The total number of transfected neurons was determined on day 2 after transfection. The numbers of alive and dead neurons were determined based on analysis of the morphology of the same neurons 4 days after transfection.

B, percentage of "time-lapse + post-hoc" revealed neurons having condensed chromatin or cleaved-caspase-3 positive staining. Neurons or their traces were visualized based on coordinates recorded on day 2 after transfection. Values normalized to the total number of transfected neurons.

C, proportions of alive and dead neurons obtained after analysis of fixed samples of cultures.

D, percentage of post-hoc fixed neurons having condensed chromatin or cleaved-caspase-3 positive staining. Values normalized to the total number of transfected neurons identified after fixation.

E, absolute numbers of alive neurons quantified in the same cultures, using time-lapse or post-hoc approaches (normalized to the number of alive neurons in time-lapse imaging approach).

F, absolute numbers of dead neurons quantified in the same samples of cultures using time-lapse or post-hoc approaches (normalized to the number of dead neurons in time-lapse imaging approach).

Supplementary Figure 5. Comparison of the ability of different KCC2-related constructs to modify chloride homeostasis in 9 div neurons. Gramicidin perforated patch. Vh=-65 mV. Transfections were performed at 7 div. *, P<0.05; **, P<0.01. 5 experiments, 1 to 3 neurons per experiment.

E_{GABA} (left plot) and times of 50% recovery of isoguvacine responses after imposed $[Cl^-]_i$ rise (right plot) in 9 div neurons transfected at 7 div with eGFP, $KCC2_{rat}$ or different mutants of eGFP-$KCC2_{rat}$. 6 experiments, 1 to 3 neurons per experiment.

To allow direct visualization of both native and mutated KCC2 proteins into neuronal cells we created a functional eGFP-fused KCC2 construct. The over-expression of this construct (called thereafter wild type eGFP-KCC2) in 9 div neurons (a developmental stage characterized by relatively depolarized E_{GABA} (Chudotvorova *et al.*, 2005) and relatively low neurons chloride extrusion capacity (Khirug *et al.*, 2005)) induced -20 mV hyperpolarizing shift of the basal level of E_{GABA} (left panel) and shortened two-fold the recovery time of E_{GABA} after experimentally imposed increase of $[Cl^-]_i$ (right panel). The detected changes of $[Cl^-]_i$ were similar to those induced by overexpression of KCC2 non-ligated to eGFP. The over-expression in same neuronal cultures of either eGFP-$KCC2_{C568A}$ or eGFP-$KCC2_{Y1087D}$ mutants modified neither basal level of E_{GABA} nor time of E_{GABA} recovery after $[Cl^-]_i$ increase **(Fig. 7*C*).** Thus, both mutants are non-functional. This conclusion is in agreement with previous reports of the non-functionality of the $KCC2_{C568A}$ (Reynolds *et al.*, 2008; Cancedda *et al.*, 2007) and $KCC2_{Y1087D}$ (Akerman & Cline, 2006) mutants expressed in neuronal cells.

Supplementary Figure 6. Neuronal survival after excitotoxic events induced by different concentrations of NMDA. Notice, in all excitotoxicity experiments, including control once, NMDA or equivalent amount of H_2O was applied together with 10 µM of glycine, a co-agonist of the NMDARs.

A, scheme of experiment. Prior to application of the NMDA culture media has been changed regularly in order to provide optimal conditions for neuronal survival.

B, Low zoom images of cultures exposed to 0 µM of NMDA (left image, control) and to 40 µM of NMDA (right image) during 60 min. After wash-out of the NMDA cultures were

grown during additional 24 hours, fixed and revealed using mouse NeuN antibody (red color), chicken MAP2 antibody (green color) and Hoescht (not shown, but see panel ***C***). In chosen examples there are similar numbers of NeuN-positive cells (9 and 8 cells in right and left images respectively). In the left image (control) all neurons are both NeuN and Map2-positive, whereas in the right image 2 neurons are Map2-positive and 6 neurons are Map2-negative. All Map2-negative neurons had condensed chromatin and were considered as dead (See **Supplementary Fig.7*B*** for statistic).

C, High zoom images of neurons from cultures illustrated in panel ***B*** at low zoom. Images in row b1 illustrate typical control neurons that were NeuN and MAP2-positive with homogeneous distribution of chromatin (indicated using white arrows). Images in rows b2 and b3 illustrate two types of NeuN-positive cells (i.e. neurons) detected in cultures 24 hours after exposure to NMDA. The first type of neurons includes healthy, Map2-positive cells with homogeneously distributed chromatin (white arrow in b2). Only such type of neurons was considered as alive in present study. The second type of NeuN-positive cells included neurons with different degrees of chromatin condensation (white arrowheads in b2 and b3). All neurons with condensed chromatin were Map2 negative and were considered as dead.

Supplementary Figure 7. Viability of neuronal cultures after exposure to NMDA.

A, Images of Map2-positive neurons exposed during 30 min to 0 (control) or 40 µM of NMDA. Cultures were fixed either 5 h or 72 hours after treatment. In overall 72 hours after treatment the density of Map2-positive neurons exposed to 40 µM of NMDA constituted 26.3±3.7% of neuronal density in control cultures (n=5). This longitudinal neuron survival test confirms that chosen excitotoxic treatment produces only partial loss of neuronal cells and thus can be used to assay both neuroprotective and neurotoxic effects of molecules of interest.

B, Dose-dependence of neuronal survival 24 hours after exposure to different concentrations of NMDA during 30 or 60 minutes. Data are normalized to values obtained with 30 min exposure to 0 µM of NMDA. Mean ± SEM. Pooled data from 5 to 6 experiments per condition. *, $P<0.05$; **, $P<0.01$; One-way ANOVA test.

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